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Targeting X-Linked Inhibitor of Apoptosis Protein to Increase the Efficacy of Endoplasmic Reticulum Stress-Induced Apoptosis for Melanoma Therapy

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Melanoma remains notoriously resistant to current chemotherapeutics, leaving an acute need for novel therapeutic approaches. The aim of this study was to determine the prognostic and therapeutic significance of X-linked inhibitor of apoptosis protein (XIAP) in melanoma through correlation of XIAP expression with disease stage, *RAS/RAF* mutational status, clinical outcome, and susceptibility to endoplasmic reticulum (ER) stress-induced cell death. XIAP expression and *N-RAS/B-RAF* mutational status were retrospectively determined in a cohort of 55 primary cutaneous melanocytic lesions selected and grouped according to the American Joint Committee on Cancer staging system. Short hairpin RNA interference of XIAP was used to analyze the effect of XIAP expression on ER stress-induced apoptosis in response to fenretinide or bortezomib *in vitro*. The results showed that XIAP positivity increased with progressive disease stage, although there was no significant correlation between XIAP positivity and combined *N-RAS/B-RAF* mutational status or clinical outcome. However, XIAP knockdown significantly increased ER stress-induced apoptosis of melanoma cells in a caspase-dependant manner. The correlation of XIAP expression with disease stage, as well as data showing that XIAP knockdown significantly increases fenretinide and bortezomib-induced apoptosis of metastatic melanoma cells, suggests that XIAP may prove to be an effective therapeutic target for melanoma therapy.

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INTRODUCTION

Malignant melanoma, the most aggressive form of skin cancer, represents a significant and growing public health burden, with an increased incidence in developed countries that has risen faster than any other malignancy over the past 40 years (Thompson *et al.*, 2005). Prognosis and patient survival is strongly associated with tumor thickness, ulceration, and extent of metastases; although early-stage tumors with a Breslow thickness of <1 mm are largely cured through surgical excision alone, patient survival rates fall once metastases occur, resulting in 5-year survival rates of $<11\%$ for those with distant metastases (Balch *et al.*, 2001). Treatment of metastatic disease is virtually futile as most melanomas at this stage are resistant to current chemo- and immunotherapy, emphasizing the acute demand for a, to our knowledge, previously unreported therapeutic approach. Resistance to current chemotherapeutic regimes likely results from the notorious resistance of most melanoma tumor cells to apoptosis (programmed cell death).

One mechanism by which melanomas may acquire resistance to apoptosis is by overexpression of one or more of the inhibitor of apoptosis protein (IAP) family. The IAPs function as important regulators of apoptosis through their inhibitory effect on caspases (*c*ysteine-dependent *asp*artyl-specific prote*ases*), the principle executioners of apoptosis (Emanuel *et al.*, 2008). In particular, X-linked inhibitor or apoptosis protein (XIAP) is overexpressed in melanoma cell lines (Kluger *et al.*, 2007) as well as in primary cutaneous and metastatic melanoma tissue (Kluger *et al.*, 2007; Emanuel *et al.*, 2008), and has therefore become increasingly relevant to the pathogenesis of melanoma (Chawla-Sarkar *et al.*, 2004; Emanuel *et al.*, 2008). XIAP is a potent inhibitor of caspases 3, 7, and 9, and is thus able to block apoptosis mediated through both extrinsic death-receptor ligation and intrinsic activation of mitochondria-mediated pathways

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Abbreviations: AJCC, American Joint Committee on Cancer; ER, endoplasmic reticulum; FFPE, formalin-fixed, paraffin-embedded; IAP, inhibitor of apoptosis protein; shRNA, short hairpin RNA; SNP, single-nucleotide polymorphism; XIAP, X-linked inhibitor of apoptosis protein

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(Schimmer *et al.*, 2006). As overexpression of XIAP in melanoma cells *in vitro* confers resistance to multiagent chemotherapy (Chawla-Sarkar *et al.*, 2004), increased levels of XIAP *in vivo* may partly explain why melanoma is resistant to apoptosis induced by current chemotherapeutic drugs. Clinically, strategies targeting XIAP are in development, and include XIAP antisense oligonucletides that are in clinical trials (Dean *et al.*, 2009), and small-molecule inhibitors that are in preclinical development (Zobel *et al.*, 2006; Vucic and Fairbrother, 2007).

Mechanisms regulating XIAP expression in melanoma are not well understood; however, recent reports have identified an indirect link between activated RAS/RAF signaling and XIAP expression (Notarbartolo *et al.*, 2005; Ueda and Richmond, 2006). Activating mutations in *N-RAS* or *B-RAF* are present in the majority of cutaneous melanomas (Davies *et al.*, 2002; Omholt *et al.*, 2002; Dong *et al.*, 2003; de Snoo and Hayward, 2005) and are implicated in melanoma development as well as resistance to apoptosis (Gray-Schopfer *et al.*, 2007). Nevertheless, the precise mechanisms by which this pathway confers chemoresistance are not defined. In addition, we have recently established that targeting the endoplasmic reticulum (ER) to generate ER stress induces apoptosis of melanoma cells both *in vitro* and *in vivo* (Lovat *et al.*, 2008; Hill *et al.*, 2009). We have identified fenretinide and bortezomib as two, to our knowledge, previously unreported ER stress-inducing agents capable of inducing apoptosis through caspase-dependent mechanisms (Lovat *et al.*, 2000; Poulaki *et al.*, 2007); nevertheless, their efficacy may be limited in melanoma by XIAP overexpression.

To validate XIAP as a therapeutic target in melanoma, the aims of this study were to evaluate the prognostic significance of XIAP expression in relation to disease stage, RAS/RAF mutational status, and clinical outcome, and to test the hypothesis that inhibition of XIAP increases the sensitivity of metastatic melanoma cells to ER stress-induced death. Defining the role of XIAP in melanoma progression and chemoresistance will therefore facilitate the design of a more effective drug combination for the clinical treatment of metastatic melanoma.

RESULTS

Clinicopathological features of patient specimens

A total of 55 melanocytic samples were collected from 25 male and 29 female patients who together had a median age of 54 years (Table 1). The majority of samples originated from the limbs and trunk, with very few from the head and neck region. Melanoma samples consisted mainly of the superficial spreading subtype, with nine nodular melanomas and one acral lentiginous melanoma. At diagnosis, the majority of patients presented with stage I or II disease; however, four patients had metastases at presentation. Subsequently, 13 patients went on to develop metastatic disease between 4 months and $5\frac{1}{2}$ years after initial presentation. The 5-year follow up data were available for the majority of melanoma cases, with 3-year follow-up data for three cases of early-stage IA disease ($\leqslant$0.5 mm Breslow thickness). In all, 11 patients died from metastatic melanoma and the remaining patients with metastatic disease were monitored for up to 7 years.

Table 1. Patient demographic data

	Number of cases
Age (years)[1]	
<54	26
⩾54	28
Gender	
Male	25
Female	29
Tumor location	
Head and neck	4
Trunk/abdomen	22
Limbs	29
Melanocytic lesions	
Benign nevi	6
In situ melanoma	7
Superficial spreading melanoma	32
Nodular melanoma	9
Acral lentiginous melanoma	1
Melanoma Breslow thickness (mm)	
0	7
<1.0	11
1.01–2.0	9
2.01–4.0	14
>4.0	8
AJCC stage	
0	7
IA and IB	20
IIA, IIB, and IIC	18
IIIA and IIIB	4
IVA, IVB, and IVC	0

Abbreviation: AJCC, American Joint Committee on Cancer, 2001.
[1]Median value.

XIAP expression in metastatic melanoma cell lines and primary melanocytes

Expression of XIAP was evaluated in primary human melanocytes and the metastatic melanoma cell lines CHL-1, A375, SK MEL-28, and WM266-4 by western blotting. Results

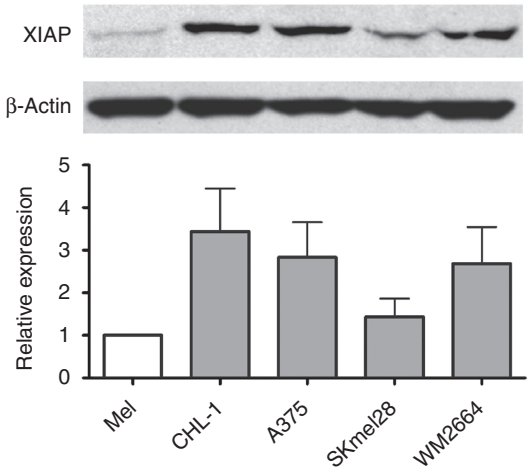

Figure 1. Expression of X-linked inhibitor of apoptosis protein (XIAP) in primary melanocytes and metastatic melanoma cell lines. (**a**) Representative western blots of XIAP and β-actin expression in primary melanocytes (1° Mel) and metastatic melanoma cell lines CHL-1, A375, SK MEL-28, and WM266-4. (**b**) Relative XIAP expression levels in primary melanocytes and metastatic melanoma cell lines quantified relative to β-actin expression to correct for loading error. Each bar is the mean ± SD of three replicate experiments.

showed that although low levels of XIAP were detectable in primary melanocytes, XIAP expression was increased in all metastatic melanoma cell lines (Figure 1).

XIAP expression in primary cutaneous melanoma and benign melanocytic nevi

To determine whether or not increased XIAP expression correlates with melanoma disease stage *in vivo*, immunohistochemistry for XIAP expression was evaluated on formalin-fixed, paraffin-embedded (FFPE) tissue derived from our cohort of 55 patient samples. XIAP expression within individual samples was indicated by deep pink staining to distinguish from brown melanin pigment often observed in melanocytic tumors; expression was compared with a no-primary antibody control from a consecutive tissue section. Where XIAP staining was detected, XIAP positivity was heterogeneous throughout the tumor, but with no consistent localization to any particular region.

Results showed a trend for increased XIAP expression in tumors, with increasing Breslow thickness being greater in superficial spreading malignant melanoma with a Breslow thickness of >2 mm compared with expression in thinner tumors and benign compound nevi (Figure 2a–d). At higher magnification, the localization of XIAP was confined in the cytoplasm of tumor cells (Figure 2i). Importantly, in the majority of samples, lymphocytes and other cells within the skin were negative for XIAP staining.

XIAP is frequently overexpressed in late-stage primary cutaneous melanoma

Analysis of XIAP staining in all 55 samples of this patient cohort, grouped according to the 2001 American Joint Committee on Cancer (AJCC) staging system, confirmed that the median XIAP positivity for each group, represented by the horizontal lines in Figure 3, increased with progressive stage. Each data point was derived from the mean percentage of cells defined visually by light microscopy as positive for XIAP, within 10 different fields of view for each sample. There was a significant effect of stage (ordinal predictor) on the percentage of XIAP-positive cells in patient samples (generalized linear model, robust estimator; Wald χ^2 122.8, 6 d.f., $P<0.001$), with stage II differing significantly from benign nevi (simple contrasts, Wald χ^2 4.182, $P<0.05$; Figure 3). Patient survival decreased with increasing stage at diagnosis (Table 1), from 70% of patients surviving with stage Ib to 25% of patients surviving with stage III. For these disease stages combined, there was no significant relationship between the percentage of XIAP-positive cells and death or survival (Mann–Whitney $U_{11,19}=87$, $P=0.45$).

Correlation of *N-RAS* and *B-RAF* mutations with clinicopathological parameters and XIAP expression

Tissue sections were single-nucleotide polymorphism (SNP) genotyped for the presence of activating mutations in *N-RAS* and *B-RAF*; of the 49 melanoma samples in this study, 3 were *N-RAS*^{G61L} mutated, 5 were *N-RAS*^{G61A} mutated, and 14 were *B-RAF*^{V600E} mutated. In addition, there was one benign nevus with an *N-RAS*^{G61L} mutation and two benign nevi with *B-RAF*^{V600E} mutations (Table 2). The *B-RAF*^{V600D} mutation was not present in any of the samples tested. Statistical comparison using Mann–Whitney analysis revealed that there was no significant relationship between mutational status (combined *N-RAS* and *B-RAF*) and XIAP positivity, either for all stages combined (Mann–Whitney $U_{25,30}=276.5$, $P=0.094$) or within AJCC stages Ib, II, or III (Mann–Whitney $U_{20,22}=178.5$, $P=0.295$).

Modulation of XIAP expression to increase ER stress-induced apoptosis in response to fenretinide and bortezomib

To test the hypothesis that inhibition of XIAP increases the efficacy of ER stress-induced cell death in response to fenretinide or bortezomib, A375 cells stably expressing XIAP short hairpin RNAs (shRNAs) were generated and their sensitivity to apoptosis was analyzed in response to 24 hours of treatment with clinically achievable concentrations of fenretinide, bortezomib, or, as a positive control for ER stress, thapsigargin. Western blotting confirmed efficient knockdown of XIAP in a clone expressing XIAP shRNA, compared with expression in untransfected cells or cells transfected with either a scrambled control sequence or an empty vector (Figure 4ai). XIAP knockdown resulted in a significant increase in apoptosis in response to fenretinide, bortezomib ($P<0.001$), and thapsigargin ($P<0.01$), compared with untransfected cells or cells transfected with a scrambled sequence (Figure 4aii, one-way analysis of variance). Furthermore, apoptosis was dependent on caspase activation, as shown by the reduction of apoptosis in the presence of the pan-caspase inhibitor carbobenzoxyl-valyl-alanyl-aspartyl-[o-methyl]-fluoromethyl-ketone and the inhibition of caspase-3 cleavage (Figure 4a and b). Similar results were also observed in CHL-1 cells (see Supplementary Figure S1 online). Conversely, clinically achievable concentrations of the current melanoma therapeutic temozolomide did not

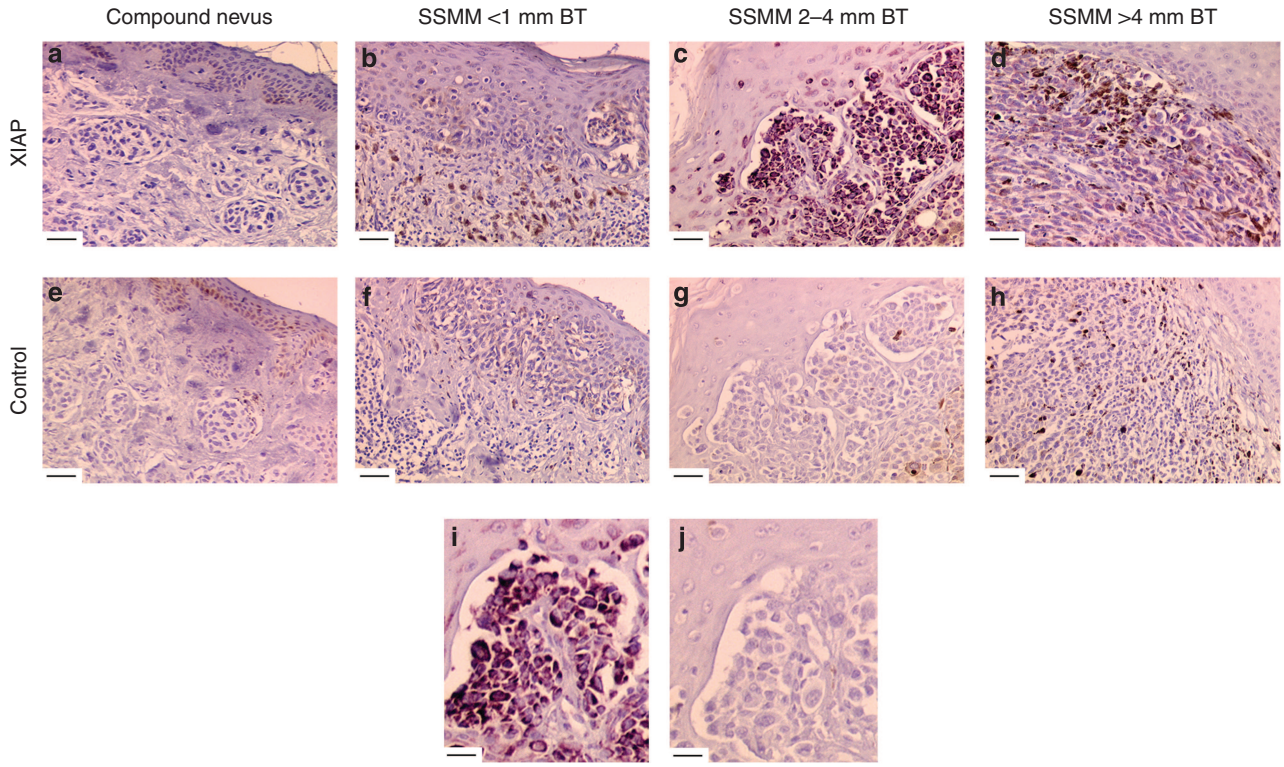

Figure 2. X-linked inhibitor of apoptosis protein (XIAP) expression in human primary cutaneous melanoma compared with controls. Immunohistochemical analysis of XIAP or control (no primary antibody) in (**a**, **e**) a compound nevus, (**b**, **f**) early-stage superficial spreading malignant melanoma (SSMM) with a Breslow thickness (BT) of <1 mm, (**c**, **g**, **i**, **j**) later-stage SSMM with a BT of 2–4 mm, and (**d**, **h**) later-stage SSMM with a BT of >4 mm. Note the white retraction artifact around several melanoma cells commonly observed after tissue fixation. Scale bars for **a–h** = 50 μm, and for **i, j** = 25 μm.

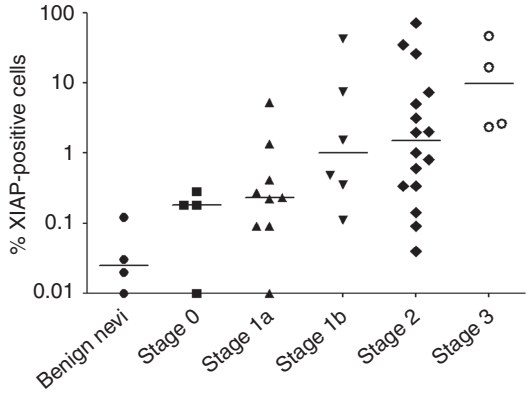

Figure 3. X-linked inhibitor of apoptosis protein (XIAP) expression increases with progressing American Joint Committee on Cancer (AJCC) disease stage. Mean XIAP-positive tumor cells (derived as a proportion of all cells within 10 fields of view) in patient cohort (55) derived from benign nevi or *in situ* melanoma (stage 0) or from AJCC stage Ia, Ib, II, and III primary cutaneous melanomas. The median percentage of XIAP positivity is represented by the horizontal line.

promote apoptosis of A375 or CHL-1 cells, either in control cells or in cells expressing XIAP shRNA (Figure 4c and Supplementary Figure S1 online).

DISCUSSION

With a growing incidence of chemotherapy-resistant malignant melanoma worldwide, a desperate need for, to our knowledge, previously unreported and more effective therapeutic strategies exists. XIAP was recently identified as a potential prognostic indicator because of its overexpression in melanoma (Kluger *et al.*, 2007; Emanuel *et al.*, 2008), and several studies have highlighted the importance of this protein for the resistance of other cancer types to chemotherapy (LaCasse *et al.*, 2006; Vogler *et al.*, 2009). In this study we determined the potential of XIAP as a therapeutic target in melanoma, and showed a significant association between increased XIAP expression and late-stage primary cutaneous melanoma, as well as the ability of XIAP knockdown to significantly enhance fenretinide and bortezomib-induced apoptosis of metastatic melanoma cells.

Retrospective selection of patient samples to include a range of thin and thick primary melanoma tumors, with a representative proportion of both nonmetastasizing and metastasizing forms, allowed for the correlation of XIAP expression with melanoma cancer stage. Consistent with previous observations, we report a trend for increased XIAP expression with increasing Breslow thickness of a tumor (Emanuel *et al.*, 2008). However, occasionally a melanoma with a thin Breslow thickness will metastasize; to additionally account for the spread of disease that is not considered by analyzing Breslow thickness alone, we investigated the correlation between XIAP expression, defined as the proportion of tumor cells positive for XIAP staining, and cancer stage according to the 2001 AJCC staging system. Our finding was that XIAP expression significantly correlated with cancer

Table 2. XIAP expression and clinicopathological characteristics

Sample	Tumor type	Breslow thickness (mm)	AJCC stage at diagnosis	Metastasis[1]	Death due to MM[2]	Mean % XIAP-positive cells[3]	*B-RAF*/ *N-RAS*[4]
1	Nevus			0	0	0.12	V600E
2	Nevus			0	0	0	G61L
3	Nevus			0	0	0.02	V600E
4	Nevus			0	0	0	
5	Nevus			0	0	0.01	
6	Nevus			0	0	0.03	
7	*In situ* MM		0	0	0	0	
8	*In situ* MM		0	0	0	0.18	
9	*In situ* MM		0	0	0	0.28	
10	*In situ* MM		0	0	0	0.01	
11	*In situ* MM		0	0	0	0	
12	*In situ* MM		0	0	0	0	
13	*In situ* MM		0	0	0	0.18	
14	SSMM	<1.0	IA	0	0	0	
15	SSMM	<1.0	IA	0	0	0.23	V600E
16	SSMM	<1.0	IA	0	0	0.09	
17	SSMM	<1.0	IA	0	0	0.09	
18	SSMM	<1.0	IA	0	0	0	G61A
19	SSMM	<1.0	IA	0	0	0	
20	SSMM	<1.0	IA	0	0	0.41	
21	SSMM	<1.0	IA	0	0	5.23	
22	SSMM	<1.0	IA	0	0	0.01	
23	SSMM	<1.0	IA	0	0	0.22	
24	SSMM	<1.0	IA	0	0	1.34	
25	SSMM	1.01–2.0	IB	0	0	0	
26	SSMM	1.01–2.0	IB	0	0	0.11	V600E
27	SSMM	1.01–2.0	IB	0	0	0	V600E
28	SSMM	1.01–2.0	IB	0	0	0.48	V600E
29	SSMM	1.01–2.0	IB	0	0	0.35	
30	SSMM	1.01–2.0	IA	0	0	0.27	
31	SSMM	2.01–4.0	IIA	0	0	0.14	V600E
32	SSMM	2.01–4.0	IIA	0	0	5.08	
33	NMM	2.01–4.0	IIA	0	0	7.3	G61L
34	SSMM	2.01–4.0	IIA	0	0	0	
35	NMM	2.01–4.0	IIA	0	0	1.02	G61L
36	NMM	>4.0	IIC	0	0	1.99	
37	NMM	>4.0	IIC	0	0	35.13	
38	SSMM	>4.0	IIC	0	0	2	
39	NM	>4.0	IIIC	1	0	46.67	V600E
40	NM	2.01–4.0	IIA	1	0	0.09	G61A
41	SSMM	2.01–4.0	IIA	1	0	71.04	V600E
42	SSMM	2.01–4.0	IIB	1	0	0.34	V600E
43	SSMM	2.01–4.0	IIA	1	0	0.81	G61A

Table 2 continued on the following page

Table 2. Continued

Sample	Tumor type	Breslow thickness (mm)	AJCC stage at diagnosis	Metastasis[1]	Death due to MM[2]	Mean % XIAP-positive cells[3]	*B-RAF*/ *N-RAS*[4]
44	ALM	2.01–4.0	IIA	1	0	3.18	
45	SSMM	1.01–2.0	IIA	1	1	0.04	G61A
46	SSMM	1.01–2.0	IB	1	1	42.5	V600E
47	SSMM	1.01–2.0	IB	1	1	1.53	V600E
48	SSMM	2.01–4.0	IIIC	1	1	16.99	V600E
49	SSMM	2.01–4.0	IIB	1	1	0	G61L
50	NM	2.01–4.0	IIA	1	1	0.34	
51	SSMM	>4	IIC	1	1	0.61	G61A
52	NM	>4	IIIC	1	1	2.35	
53	NM	2.01–4.0	IB	1	1	7.47	V600E
54	SSMM	>4	IIC	1	1	26.16	V600E
55	NM	>4	IIIB	1	1	2.65	V600E

Abbreviations: AJCC, 2001 American Joint Committee on Cancer; ALM, acral lentiginous melanoma; MM, malignant melanoma; NM, nodular melanoma; SSMM, superficial spreading malignant melanoma; XIAP, X-linked inhibitor of apoptosis protein.
[1]Metastasis: 0, patient did not develop metastases during follow-up period; 1, patient went on to develop metastases after diagnosis.
[2]Death due to MM: 0, patient did not die from disease; 1, patient died from melanoma after diagnosis.
[3]Mean % XIAP-positive cells: the percentage of tumor cells expressing XIAP (mean of 10 fields of view).
[4]*B-RAF*/*N-RAS* SNP mutations: *B-RAF* included V600E and V600D, and *N-RAS* included G61L and G61A.

stage, suggesting that XIAP is a useful prognostic marker. This analysis did not correlate primary tumors that subsequently metastasized, which needs to be taken into consideration to fully evaluate XIAP expression as a prognostic marker.

Oncogenic RAS/RAF signaling is now well characterized as a mechanism contributing to the establishment of melanoma (Fecher *et al.*, 2007). Mutations that activate either RAS or RAF, in particular *N-RAS*$^{G61L/A}$ and *B-RAF*^{V600E}, have been identified in a large proportion of melanomas and are implicated in melanoma development and resistance to apoptosis (Gorden *et al.*, 2003; Garnett and Marais, 2004). Recent reports identifying XIAP as an indirect target of RAS/RAF signaling suggest that XIAP upregulation in melanoma may be related to the presence of oncogenic mutations in this pathway (Notarbartolo *et al.*, 2005; Ueda and Richmond, 2006). To test this hypothesis, SNP genotyping assays were used to identify the most commonly occurring *N-RAS* and *B-RAF* mutations in tissue from primary melanoma samples. There was no significant correlation between the *N-RAS* or *B-RAF* mutational status of tumors and the level of XIAP expression; nevertheless, we cannot exclude the possibility that other rare activating mutations influencing RAS/RAF signaling may have been present that could influence XIAP expression.

The findings of a significant association of XIAP expression with poor prognosis, as well as data showing that XIAP knockdown significantly increases fenretinide and bortezomib-induced apoptosis of metastatic melanoma cells in a caspase-dependent manner, suggests that XIAP may prove to be an effective therapeutic target for melanoma treatment. A recent clinical trial of AEG35156 (Aegera Therapeutics; Schimmer *et al.*, 2009) for the treatment of acute myeloid leukemia showed that XIAP inhibition was generally well tolerated, therefore suggesting that combining an ER stress-inducing agent, such as fenretinide or bortezomib, with an inhibitor of XIAP or a small-molecule antagonist (Vogler *et al.*, 2009) could represent a more effective therapeutic strategy for the treatment of metastatic melanoma.

MATERIALS AND METHODS

Patients and specimens

All cases were selected retrospectively from the Department of Pathology at the Royal Victoria Infirmary, Newcastle Upon Tyne, UK, and consisted of a total of 55 melanocytic lesions, including benign nevi ($n=6$) and primary cutaneous malignant melanomas ($n=49$; ranging from Breslow thickness 0.2 to 9.0 mm and Clark level I–V), with or without ulceration (Table 1). Cases were selected to encompass a range of disease stages (as defined by the 2001 AJCC), and for which clinical and histopathological follow-up was available (ranging from 0.5 to 7.0 years). Cases included thin, intermediate, and thick nonmetastasizing primary tumors, as well as intermediate and thick primary tumors, all of which proceeded to metastasize. Archival FFPE tissue blocks were collected after ethical approval from the research ethics committee of Northumberland National Health Service and with written, informed patient consent obtained as appropriate, and the study was performed in accordance with the Declaration of Helsinki Principles. Exclusion criteria included patients of age <16 years, pregnancy at the time of surgical treatment, and those with an immune-modifying disease or who had used immune-modifying drugs within 3 months of their surgical treatment. Clinical end point was time to disease-specific death.

Evaluation of activating mutations of *N-RAS* and *B-RAF* in the patient cohort

DNA was extracted from FFPE tissue blocks using QIAamp DNA FFPE Tissue Kit (Qiagen, Crawley, UK) according to the

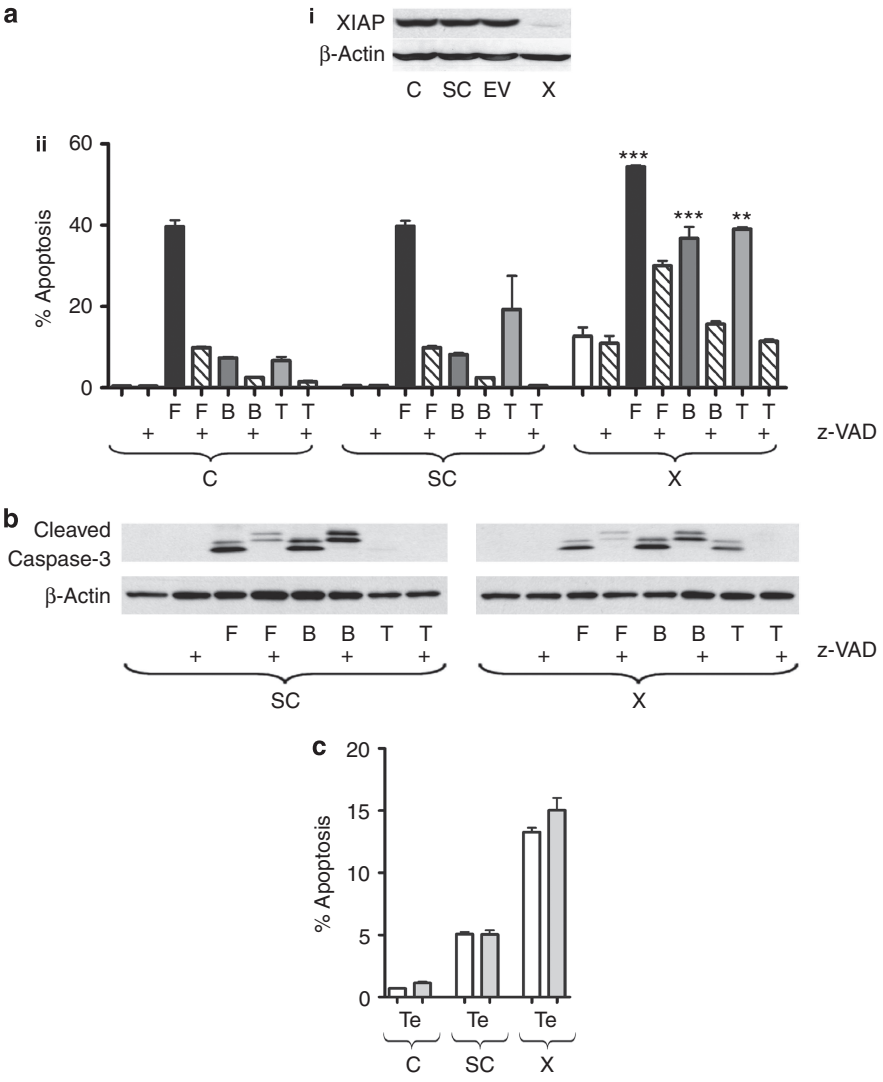

Figure 4. X-linked inhibitor of apoptosis protein (XIAP) inhibits endoplasmic reticulum (ER) stress-induced apoptosis in a caspase-dependent manner. A375 cells were untransfected (C) or transfected with either a scrambled control sequence (SC), empty vector (EV), or XIAP shRNA (X) before treatment with 12 μM fenretinide (F), 200 nM bortezomib (B), or 7.5 μM thapsigargin (T) in the presence or absence of 25 μM z-VAD for 24 hours. (**a**) Percentage of apoptosis; each bar is the mean of three replicates ± SD; $^{**}P<0.01$, $^{***}P<0.001$. (ii) Western blots for XIAP or β-actin expression. (**b**) Western blots for cleaved caspase-3 or β-actin expression. (**c**) A375 cells (as above) were treated with 50 μM temozolomide (Te) for 24 hours. Data are expressed as percentage of apoptosis; each bar is the mean of three replicates ± SD.

manufacturer's protocol. Immediate tissue sections to those taken for routine histology were taken for DNA extraction. PCR amplification and sequencing of the two most common *N-RAS* codon-61 SNPs on exon 2, *N-RAS* G61L and G61A, as well as the SNPs for *B-RAF* V600D and V600E mutations (Davies *et al.*, 2002; Omholt *et al.*, 2002) were performed using custom-made primers and probes (see Supplementary Table S1 online; Custom TaqMan SNP Genotyping Assay; Applied Biosystems, Warrington, UK). Quantitative PCR was performed with Chromo4 (MJ/Bio-Rad, Hemel Hempstead, Hertfordshire, UK), using Faststart Universal Probe Master (Roche Diagnostics, Burgess Hill, UK).

XIAP immunohistochemistry

Patient tumor specimens (6 to 7 μm consecutive FFPE tissue sections) were placed onto (3-aminopropyl)triethoxysilane (APES; Sigma-Aldrich, Dorset, UK)-coated glass slides and baked overnight at 37 °C. Sections were deparaffinized in Histo-Clear (30 minutes; Sigma-Aldrich) and rehydrated in a graded series of ethanol before H_2O. Slides were heated at 100 °C in a microwave oven in 0.01 M Tris-HCl pH 7.6 for 20 minutes and cooled to room temperature. After permeabilization for 10 minutes with 0.2% Triton X-100 in phosphate-buffered saline, sections were then incubated in 3% hydrogen peroxide in water for 10 minutes. After incubation with 5% horse serum in phosphate-buffered saline containing 0.05% Tween-20 for 20 minutes at room temperature, sections were incubated with monoclonal anti-XIAP (BD Biosciences, Oxford, UK) diluted 1:250 in phosphate-buffered saline with 2% albumin and 5% horse serum at 4 °C for 72 hours (Emanuel *et al.*, 2008). Antibody detection was performed using a mouse IgG ABC *elite* Vectastain Kit (Vector Labs, Peterborough, UK) and developed with Vector VIP to give a deep-pink stain (Vector Labs). Finally, sections were counterstained using hematoxylin, dehydrated in increasing concentrations of ethanol

solution, then cleaned in Histo-Clear, and mounted in di'n'butyl phthalate in xylene before visualization.

All immunostained slides were coded so that clinicopathological data could not be directly correlated. Staining was visualized by light microscopy; 10 fields from XIAP-stained sections were systematically selected within the tumor, and images were captured and analyzed using Leica QWin software (Leica Microsystems, Newcastle upon Tyne, UK). The number of positively stained cells and the total number of cells were counted to allow calculation of the proportion of tumor cells staining positive.

Cell culture, drug treatment, and analysis of apoptosis

Melanoma cell lines, CHL-1, A375, and WM266-4 (ATCC), were cultured in DMEM (Lonza, Cambridge, UK) supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C/5% CO_2 as previously described (Corazzari *et al.*, 2007). Melanocytes were obtained from human foreskin, isolated from fibroblasts and keratinocytes by selective trypsinization (Todd and Reynolds, 1998), confirmed by immunostaining for the melanocyte differentiation antigen Melan-A (Abcam plc, Cambridge, UK), and cultured in medium 254 supplemented with human melanocyte growth supplement-2 according to the manufacturer's specifications (Invitrogen, Paisley, UK). Fenretinide (Janssen-Cilag High Wycombe, UK), bortezomib (Janssen-Cilag), thapsigargin (Sigma-Aldrich), temozolomide (OSI Pharmaceuticals, Oxford, UK), or carbobenzoxyl-valyl-alanyl-aspartyl-[o-methyl]-fluoromethyl-ketone (Promega, Southampton, UK) were added to cells in ethanol (fenretinide) or DMSO (bortezomib, thapsigargin, or carbobenzoxyl-valyl-alanyl-aspartyl-[o-methyl]-fluoromethyl-ketone) with an equal volume of vehicle used to treat control cells. Flow cytometry of propidium iodide-stained cells was used to estimate the level of cell death or apoptosis by measuring the percentage of cells in the sub-G1 fraction (Corazzari *et al.*, 2007).

Retroviral transfection for shRNA interference of XIAP

For shRNA-expressing vectors against XIAP, the target sequence 5′-GTGGTAGTCCTGTTTCAGC-3′ (Burstein *et al.*, 2004) was incorporated into the following oligos: fw, 5′-GATCCCGTGGTAGTCCTGTTTCAGCTTCAAGAGAGCTGAAACAGGACTACCACTTTTTGGAAA-3′ and rev, 5′-AGCTTTTCCAAAAAGTGGTAGTCCTGTTTCAGCTCTCTTGAAGCTGAAACAGGACTACCACGGG-3′. Oligos were annealed and cloned into the pRETROSUPER vector (Brummelkamp *et al.*, 2002) using *Bgl*II and *Hin*dIII, propagated in supercompetent XL1-Blue *E. coli* cells (Stratagene, Stockport, UK) and isolated using a HiSpeed plasmid Maxi kit (Qiagen) according to the manufacturer's instructions. Using lipofectamine 2000 (Invitrogen), 1 μg of purified plasmid was transfected into pRT67 mouse fibroblast packaging cells to produce live virus. Resulting supernatant containing the virus was then used to transduce A375 and CHL-1 human melanoma cells and stable clones were selected using 1 μg ml^{-1} puromycin (Invitrogen).

WESTERN BLOTTING

Total protein was extracted from cell pellets into lysis buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 25 mM NaF, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM Na_3VO_4, and complete protease inhibitor cocktail (Roche Diagnostics), separated by electrophoresis through 4–20% SDS-PAGE gels (20 μg per lane; Invitrogen), and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK) as previously described (Armstrong *et al.*, 2007). Blots were probed with antibodies to XIAP (Cell Signaling Technology, Hitchin, UK; diluted 1:1,000), cleaved caspase-3 (Cell Signaling Technology; diluted 1:1,000), and β-actin (Sigma-Aldrich; diluted 1:5,000). The binding of primary antibodies was detected with secondary peroxidase-conjugated antibodies (Upstate Biotechnology, Vector Labs; diluted 1:2000) and visualized using the enhanced chemiluminescence plus system (Amersham Biosciences).

Statistical analysis

Statistical analyses were carried out using SPSS version 15 (SPSS, Chicago, IL) or Graphpad Prism version 5 (Graphpad Software, La Jolla, CA). In the immunohistochemistry studies, data for the percentage of XIAP-positive cells with AJCC stage as ordinal predictor were analyzed with a generalized linear model using a log link function and normal distribution with a robust estimator; simple contrasts were used to compare each stage with data for the first category in the analysis (benign nevus). An intercept (not significant in the initial analysis) was not included in the final model (fitted model compared with null model: likelihood ratio χ^2 13.335, 6 d.f., $P = 0.038$). Relationships between XIAP positivity and *N-RAS/B-RAF* mutational status were examined using Mann–Whitney tests. Data for apoptosis in cells transfected with XIAP shRNA were analyzed by one-way analysis of variance with *post hoc* Bonferroni test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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